Microbial communities associated with *Pythium arrhenomanes* lesions on the roots of *Zea mays*

Undergraduate Research Project by

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Soil was collected from a no-till, corn-corn rotation plot in Northwest Ohio for use in a greenhouse assay. Corn (*Zea mays* L.) plants were grown in sterile and non-sterile soil, amended with the plant pathogenic fungus *Pythium arrhenomanes* (Drechsler) according to a two by two factorial. After four weeks, plants grown in non-sterilized soil infested with *P. arrhenomanes* showed a significantly (P=0.05) lower incidence of Pythium root rot as compared to plants grown in *P. arrhenomanes* infested sterile soil.

The suppression of Pythium root rot in natural soils prompted a comparison of bacterial communities associated with lesioned and asymptomatic roots. Rhizosphere bacteria were isolated from asymptomatic and lesioned root segments of *Z. mays* grown in natural soil using 0.001 strength Trypticase Soy Broth Agar (TSBA). Approximately 100 bacteria from each community were identified via gas chromatographic fatty acid methyl ester (GC-FAME) analysis. A single replication of each treatment was completed. As a result, conclusions are limited to the root samples examined and may not be representative of the natural setting. The bacterial species diversity of culturable taxa indicated a 30% similarity of communities from asymptomatic and lesioned roots. While 54% and 19% of all strains isolated from asymptomatic roots belonged to the genuses *Kluyvera* and *Pseudomonas* respectively, these genuses comprised only 23% and 18% of the strains isolated from lesioned roots.

Bacterial strains were screened for the ability to suppress growth of *P. arrhenomanes* on the low iron medium, King's media B. Ten and 14% of the strains from asymptomatic and lesioned roots, respectively, suppressed the growth of *P. arrhenomanes*. Suppression by these organisms was apparently due to the action of diffusable antifungal metabolites. The most prevalent and efficacious strains were classified as *Pseudomonas putida* biotype B and *Kluyvera* spp.

Approximately 21% of strains isolated from asymptomatic roots and 50% of the strains isolated from lesioned roots were hyperparasitic to *P. arrhenomanes in vitro*. *Kluyvera cryocrescens*, *Kluyvera* spp. and a few strains of *Pseudomonas rubrisalbicans* were the most efficacious and prevalent hyperparasites *in vitro*. In the samples studied, more strains antagonistic to *P. arrhenomanes in vitro* were isolated from lesions than from asymptomatic roots.

Introduction

Oomycetous fungi such as *Pythium* spp. are the causative agents of root rots and damping-off diseases of many crops (10). Pythium root rot is a primary cause of yield loss in *Zea mays* production in Northwest Ohio. Yield losses are particularly pronounced in poorly drained soil(4).

Much research has focused on the isolation of soil microorganisms capable of controlling Pythium diseases (12). Such varied microorganisms as the ascomycete *Chaetomium globosum* (5), the deuteromycete *Gliocladium virens* (14) and bacteria such as *Burkholderia cepacia* (7), *Pseudomonas fluorescens* (9), *P. putida* (11), *P. aeruginosa* (2), *Arthrobacter* spp.(6), and *Enterobacter cloacae*(6) have been implicated as biocontrol agents for Pythium root rot and damping-off.

Mechanisms of biocontrol are generally characterized as antibiosis, competition, hyperparasitism and induced systemic resistance(3). Antibiosis is generally caused by diffusable secondary metabolites which cause inhibition or death of competing microorganisms. Other secondary metabolites, such as siderophores, which are iron chelating compounds produced under iron-limiting conditions, may also inhibit soilborne fungi(11). Recent studies have found a lack of evidence for the role of antifungal metabolites such as pyoveridine (a siderophore), pyoluteorin, pyrrolnitrin, and cyanide in *in situ* suppression of Pythium damping-off, as knock-out mutations for these metabolites do not affect a strain's suppressive functions (9).

Competition for niches and other limited nutrients in the rhizosphere may be another mechanism of biocontrol (15). Many bacteria and fungi have been shown to produce hydrolytic enzymes after induction by appropriate substrates. Hyperparasitic interactions and suppression of *Pythium* spp. by

Arthrobacter spp. and *Enterobacter cloacae* are thought to be caused by lytic capabilities of these organisms.(3).

The objectives of this study were to 1) determine the relationship between lesion development and overall plant health as measured by shoot height and shoot and root dry weight, 2) determine whether indigenous microflora suppress root lesion development and 3) assuming that the microflora induce suppression, determine which species of culturable microorganisms are responsible for the suppressive effect.

Materials and Methods

Preparation of inocula. *P. arrhenomanes* isolates 201-25 and 402-20(Deep), isolated from corn root lesions, were maintained on modified sucrose-asparagine medium (modified SAPCNB) (2.5 g sucrose, 0.27 g asparagine, 0.15 g KH_2PO_4, 0.15 g K_2HPO_4, 0.19 g $MgSO_47H_2O$, 0.08 g $CaCl_2$,19.0 g Bacto agar, and 1 L distilled H_20)(13). SAPCNB medium was modified by leaving out two fungicides, 0.027 g 75% active pentachloronitrobenzene (PCNB) Terrachlor, and 0.02 g 50% benomyl. Inoculum was prepared by growing isolates in potato soil medium. This medium was prepared by mixing 500 cm^3 of sifted greenhouse loam soil, 50 g chopped potato pieces, and 50 ml of distilled water by hand. The medium was autoclaved for one hour on two consecutive days. Two 1-L Erlenmeyer flasks containing potato soil media were then inoculated with 5 plugs of isolates 201-25 and 402-20, respectively, and an additional 25 ml of sterile water was also added. Inoculum was incubated at room temperature for five days and shaken daily(4).

Virulence test. Soil was gathered from a Hoytville, Ohio corn-corn rotation no-till plot. Half of the soil was autoclaved for 12 hours. The 201-25 and 402-20 inocula were thoroughly mixed together by hand. This mixture was used to inoculate non-sterilized soil and sterilized soil. Ten 11.5 cm pots were used for each treatment: 1) non-sterilized non-inoculated, 2) non-sterilized inoculated, 3) sterilized non-inoculated, and 4) sterilized inoculated soil. Four seeds of *Zea mays* Pioneer 3394 were planted in each pot.

All pots were kept well watered in a greenhouse for 4 weeks. Shoot height was measured weekly. After four weeks, shoots were cut at the soil line and dried. Roots were washed in tap water to removed soil. Many fine roots

were lost, but the major roots were retained. Five lesioned root pieces from each plant were plated on regular SAPCNB media (modified SAPCNB, containing 0.027 g 75% active pentachloronitrobenzene (PNCB) Tetrachlor, and 0.02 g 50% benomyl) (13). After four days, root pieces were examined microscopically for growth of *P. arrhenomanes*. The relative health of each plant was assessed with a root health index (RHI) in which: 5= asymptomatic; 4= 3 root pieces infected with P. arrhenomanes; 3= 4 infected root pieces; 2= 5 infected root pieces; and 1= pre-emergence damping-off. The remaining root mass was also dried, and the dry weights of both stems and roots recorded.

Statistical analysis. A one-way analysis of variance was performed by using Minitab statistical software (Minitab Inc., State College, Pa.) Separations of means were based on the least-significant difference (P= 0.05)(1). The level of similarity between bacterial species in asymptomatic and lesioned root samples was determined using Dice similarity coefficients, calculated as follows: $(2a/(2a+b+c)$, in which a is the number of common taxa in the two samples , b is the number of unique taxa which occur in the first but not the second sample, and c is the number of unique taxa which occur in the second, but not the first sample (1).

Microbial community analysis

Isolation of bacteria. *Z. mays* plants (Pioneer 3394) were grown for four weeks in 11.5 cm pots of native soil from Northwestern Ohio. This soil has been shown to be heavily infested with *P. arrhenomanes* (4). The root mass was removed from each pot and placed in a beaker containing 500 ml sterile phosphate buffer (6.25 g KH_2PO_4, 11.8 g $K_2HPO_43H_2O$ 1 L distilled H_20, pH adjusted to 7.0) (1). The root mass was sonicated for 10 min to remove loosely

associated soil. The phosphate buffer was replaced and the process repeated three times, until the majority of soil was removed from the roots. Three 200-μg samples of both lesioned and asymptomatic roots were randomly selected. Each sample was then placed in a Ten Broeck tissue homogenizer with 150 μL of sterile phosphate buffer. Dilutions of 10^{-2}, 10^{-3}, and 10^{-4} were then plated in triplicate onto three media 1) 0.1 Trypticase Soy Broth Agar (TSBA), 2) 0.001 TSBA, and 3) Kings media B (1.5 g K_2HPO_4, 1.5 g $MgSO_4$, 10 ml glycerol, 20 g Difco proteose peptone #3, 17 g Bacto agar, adjusted to pH 7.2) (11).

Enumeration and purification. At two and five days after plating, colonies on each plate were enumerated, and the number of colony forming units per gram dry weight root was determined. One hundred colonies from one 0.001 TSBA plate from each of the healthy and lesioned roots were collected and transferred to 0.001 TSBA plates. These colonies were then purified by repeated streaking on 0.001 TSBA media and were stored at -80 C in Nutrient Broth amended with 15% glycerol.

Identification. Isolates were identified via gc-FAME analysis with a model HP6958 Microbial Identification System apparatus equipped with version 6 of the Aerobic Library (Microbial ID, Inc., Newark DE.) according to the manufacturers set procedures. Strains with a similarity index greater than 0.6 were assigned to a genus, strains with a similarity index between 0.6 and 0.3 were assigned to a species, and strains with a similarity index of less than 0.3 were assigned to GC similarity groups on the basis of fatty acid profile similarities.

***In vitro* Biocontrol assays.** King's medium B plates were inoculated with individual bacterial strains. After a two-day incubation one plug, 0.6-cm in diameter, of *P. arrhenomanes* culture, grown on modified SAPCNB medium was placed 5 cm from the bacterial streak. Hyphal and bacterial growth were measured and recorded after two and five days of further incubation.

Results

Z. mays grown in sterilized non-inoculated soil had significantly (P=0.05) greater shoot and root dry weight as compared to all other treatments (Table 1). The root dry weights of plants grown in non-sterilized inoculated, non-sterilized non-inoculated, and sterilized inoculated treatments did not differ. The shoot dry weights of the non-sterile non-inoculated, and non-sterile inoculated treatments were similar but were significantly (P=0.05) higher than the shoot dry weights of the sterile inoculated treatment. Shoot heights were similar in all treatments.

Roots of *Z. mays* grown in sterilized non-inoculated soil had significantly (P=0.05) higher RHI values as compared to all other treatments. RHIs for plants grown in sterilized inoculated controls were significantly (P=0.05) lower than all other treatments. The root health of plants grown in non-sterilized non-inoculated and non-sterilized inoculated soils did not differ.

Colony forming units on 0.001 TSBA averaged 2.55×10^7 and 1.54×10^7 per gram dry weight root for lesioned and asymptomatic roots, respectively. Bacterial strains isolated from asymptomatic and lesioned roots were 30% similar. *Kluyvera* spp. comprised 54% and 18.9% of the bacterial community in asymptomatic and lesioned roots, respectively (Table 2). The next most prominent genus, *Pseudomonas* spp. comprised 19% and 18% of the isolated strains from the asymptomatic and lesioned roots, respectively.

Ten percent of bacterial strains isolated from asymptomatic roots and 14% of strains isolated from lesioned roots suppressed the growth of *P. arrhenomanes* due to the apparent action of diffusable antibiotic metabolites. Hyperparasitism to *P. arrhenomanes* was observed in 21% and 50% of the strains isolated from asymptomatic and lesioned roots, respectively. These isolates displayed little or no suppression of fungal growth when not in

contact with mycelium. Once contact occurred however, they rapidly colonized the mycelium, suggesting a possible lytic effect.

Discussion

There exists a clear correlation between the increase in the number of root lesions and the decrease in plant health based on the measurements of root and shoot dry weight and RHI. An exception to this observation is that there exists no significant relationship between shoot height and the number of root lesions. This contradiction may reflect low quality or amount of light due to minimal row spacing or greenhouse cultivation.

The plants grown in sterilized inoculated soil had significantly (P=0.05) lower shoot dry weights, as well as significantly (P=0.05) lower root health indexes than plants in non-sterile inoculated treatments. Natural soil, therefore, had the ability to suppress Pythium root rot, although this property was destroyed upon heat sterilization. The suppressing property could be chemical, physical, or biological.

The hypothesis that antagonistic interactions between microflora were responsible for the death of *P. arrhenomanes* in root lesions was supported by the observation that more strains antagonistic to *P. arrhenomanes in vitro* were isolated from lesioned roots as compared to asymptomatic roots. Some of the bacterial strains found to be antagonistic to *P. arrhenomanes in vitro* were similar to taxa previously identified as biocontrol agents of *Pythium* spp. These taxa include *Pseudomonas putida*, and *Berkholderia cepacia*. Antagonistic strains not previously identified as *Pythium* spp. biocontrol agents were assigned to the taxa *Enterobacter intermedius*, *E. taylorae*, *Pseudomonas marginelis*, *P. rubrisubalbicans*, *Kluyvera cryocrescens*, *Kluyvera* spp., and *Stenotrophomonas* spp.

Further studies should include *in situ* assays with strains antagonistic to *P. arrhenomanes in vitro* to determine the efficacy of these isolates as field biocontrol agents. Also, generalizations about the bacterial community

composition associated with asymptomatic and healthy roots are limited to the two samples examined. Further studies involving more repetitions would allow for broader generalizations comparing the bacterial communities in these niches.

This study was a preliminary look at the bacteria antagonistic to *P. arrhenomanes* in the rhizosphere. Future studies could include an examination of the mechanism of community suppression of disease. Further community analyses could examine the changes in asymptomatic and lesioned root microflora composition under conditions of changing water potential or in conventional till versus no-till systems.

Table 1. Measurements of plant health including average shoot height, root health index, root dry weight, and shoot dry weight for four soil treatments.

Treatment	Shoot height (cm)	Root Health[A] Index (RHI)	Root dry Weight (g)	Shoot dry Weight (g)
1. non-sterile, non-inoculated	79.8	2.6	0.66	1.51
2. non-sterile, inoculated	80.1	2.4	0.65	1.40
3. sterile, non-inoculated	78.1	5	0.97	1.79
4. sterile, inoculated	76.2	1.7	0.55	1.11
LSD(0.05)	4.24	0.428	0.154	0.256

[A] The relative health of each plant was assessed with a root health index (RHI) in which: 5= asymptomatic; 4= 3 root pieces infected with *P. arrhenomanes*; 3= 4 infected root pieces; 2= 5 infected root pieces; and 1= pre emergence damping off.

Table 2. Relative population densities of bacteria isolated on 0.0001 TSBA from asymptomatic and lesioned *Z. mays* roots grown in natural soil.

Taxon[A]	Relative population density[B] asymptomatic	lesioned
Acidovorax delafieldii	1	
Acidovorax spp.	1	0.9
Agrobacterium radiobacter		2.7
Azospirillum brasilense	1	
Bergeyella zoohelcum		1
Berkholderia cepacia	1	
Berkholderia pickettii		3.6
Chryseobacterium balulstinum		0.9
Chryseobacterium indologens		0.9
Chryseobacterium sp.		0.9
Citrobacter freundii		0.9
Comamonas acidovorans		7.2
Enterobacter asburiae		3.6
Enterobacter taylorae	1	
Enterobacter intermedius	3	
Enterobacter sp.	1	
Escherichia coli	1	
Kluyvera cryocrescens	39	16.2
Kluyvera spp.	15	2.7
Orthrobactrum anthropi		0.9
Pseudomonas marginalis		0.9
Pseudomonas putida	3	8.1
Pseudomonas rubrisubalbicans	15	16.2
Pseudomonas syringae	1	
Pseudomonas spp.	4	2.7
Pantoea agglomerans		1.8
Pantoea ananas	1	
Stenotrophomonas sp.	1	
Vibrio cholerae Ogawa	1	
Vibrio cholerae	1	
GC similarity group I		3.6
GC similarity group II		4.5
GC similarity group III		2.7
GC similarity group IV		2.7
GC similarity group V		2.7
GC similarity group VI		1.8
unidentifiable[C]	2	1.9
lost[D]	8	7.2

[A]Isolates were identified via gc-FAME analysis. Strains with a similarity index greater than 0.6 were assigned to a genus, strains with a similarity index between 0.6 and 0.3 were assigned to a species, and strains with a similarity index of less than 0.3 were assigned to GC similarity groups on the basis of fatty acid profile similarities.

[B] Relative population densities are expressed as the number of strains assigned to a taxon as a percentage of all strains from that sample.

[C] Strains unculturable on media or temperature necessary for identification using the Microbial Identification System

[D] Strains lost upon subculturing

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